DEVELOPMENT OF EYE COLORS IN DROSOPHILA: SOME PROPERTIES OF THE HORMONES CONCERNED*

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Diffusible substances showing hormone-like action in modifying the development of eye color are known in *Drosophila* and in certain other insects. For details of this work and references to the literature see Kühn (1937), Ephrussi (1938), and Beadle, Anderson, and Maxwell (1937). These substances are active when injected into, or fed to, appropriate test larvae. The so called v^+ substance causes a modification of the eye color of vermilion brown (*v bw*) animals from a pale pink towards brown. Similarly, cn^+ substance induces a modification of cinnabar brown (*cn bw*) eye color from colorless towards brown.

Chemical investigations of the eye color hormones have been carried out by Khouvine and Ephrussi (1937), Thimann and Beadle (1937), and Becker (1937). Similar investigations have been continued with the aim of eventually isolating and identifying these hormones. This paper reports some of the further information which has been obtained primarily in regard to the properties, chemical nature, and possible methods of purification of the v^+ hormone.

Source of the Hormone and Methods of Testing

The v^+ hormone used for this work was obtained from 24–48 hour old wild type pupae. The pupae were washed and dried at 100°C. for 1 hour. This served both to inactivate the enzymes and to dry the material. The dried pupae were kept under reduced pressure until needed. The hormone was extracted from finely ground dried pupae by procedures to be described later in this paper. Small aliquots of the extracts to be tested were dried under reduced pressure at 100°C.,

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weighed, and dissolved in Ringer's solution and, if necessary, the pH adjusted to 6.0. In most cases the concentration of extract in the test solutions did not exceed 0.7 per cent. The solutions prepared in this way were sealed in small glass tubes and immersed in boiling water for 10 minutes. This was found to be necessary to prevent bacterial growth and consequent toxicity for the test animals. Solutions prepared in this way retained their activity indefinitely.

The activity of the various solutions was determined by injecting them into the body cavities of *v bw* larvae shortly before puparium formation. The effect manifested itself in a modification of the eye color from nearly colorless towards brown. Ten to fifteen larvae were injected with each solution to be tested.

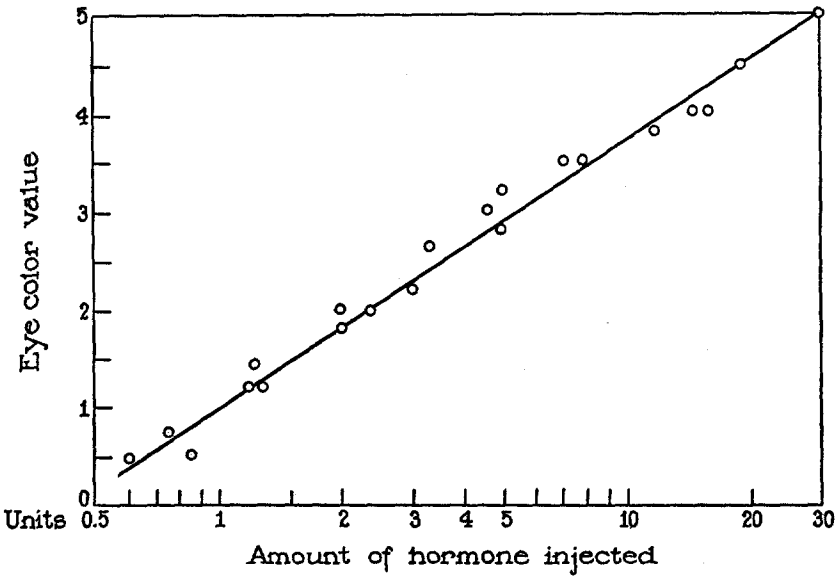

FIG. 1. Effect of concentration of v^+ hormone on eye color of *v bw* flies.

Quantitative Determination of v^+ Hormone.—The development of a reasonably accurate method of determining the amount of the hormone in a given extract was necessary for exact chemical work. Dilutions of active extracts were injected into the test larvae, and the intensity of the induced eye color was graded in terms of the arbitrary color values introduced by Thimann and Beadle. The numerical values on this scale range from 0 (no response) to 5 (maximum response indistinguishable from brown control flies). In this and subsequent determinations approximately 1.4 $mm.^3$ (the maximum volume practicable) was injected into each larva, and the maximum effect in each series of injections was taken as the best measure of the activity of that solution. The results of several such dilution series are shown in Fig. 1, where the abscissa represents the amounts injected,

plotted on a logarithmic scale, and the ordinates the arbitrary color values from 0 to 5. It is evident that, when plotted in this way, the relation is approximately linear. To facilitate calculations, an arbitrary unit of activity was selected and defined as that amount of the hormone which when injected into one larva brings about a modification of eye color to a value of 1.0 on the color scale. In order to facilitate grading of color values and to ensure reproducible results, certain genetic types of flies with different but constant eye color values were selected to form a set of standards. All test animals were compared and graded by comparison with the standard flies. The genetic composition and the eye color values of

TABLE I

Genetic Constitution and Color Values of Flies Used As Standards

Constitution	Color value
Vermilion; brown (*v; bw*)*	0.0
Suppressor-1 of vermilion, vermilion/vermilion; brown (*su*^{1}—*v v/v; bw*) ♀	0.5
Suppressor-2 of vermilion, vermilion; brown (*su*^{2}—*v, v; bw*) ♂	1.0
Suppressor-2 of vermilion, vermilion; brown (*su*^{2}—*v, v; bw*) ♀	2.0
Brown; claret (*bw; ca*)† ♀ or ♂	2.0
Eosin (*w*^{e})‡ ♂	3.0
Suppressor-1 of vermilion, vermilion; brown (*su*^{1}—*v, v; bw*) ♂	3.5
Eosin (*w*^{e}) ♀	4.7
Brown (*bw*) ♀ or ♂	5.0

* Females of the constitution vermilion brown are somewhat darker than males. Because of this, it is desirable in critical tests to compare flies of the same sex.

† Flies of this combination have an eye color of an intensity corresponding to the color value 2, but the shade of color is not quite that desired; this standard, used in most of the work reported in this paper, probably will be replaced in future work.

‡ To a lesser extent, the same objection applies to this standard as to the brown claret combination—see footnote above.

these standards are shown in Table I. The age of these standards must be controlled, as the color varies slightly with age. To ensure the greatest accuracy in determination, most solutions were diluted so that a color between 1.0 and 2.0 was induced.

Extraction of the Hormone.—It has previously been shown (Khouvine and Ephrussi, 1937; and Thimann and Beadle, 1937) that the eye color hormones may be extracted from pupae with water or with alcohol-ether mixtures. Various procedures were tried for the extraction of the v^+ hormone from dried wild type pupae, and the following method was found most successful. The ground dried pupae were extracted several times with boiling chloroform, and this inactive

extract was discarded. The hormone was then extracted by repeated treatments with hot 95 per cent ethyl alcohol. The alcohol was removed from the yellow extract under reduced pressure and the residue taken up in distilled water. The water-insoluble portion of this mixture was removed by centrifuging. The final product was a fairly clear yellow solution. The results of a number of extractions are summarized in Table II. Approximately 5 per cent of the original weight of the pupae were contained in the final extract. Disregarding a few inexplicable cases in which extracts of quite low activity were obtained, the average activity was about 280,000 units per gram extract. This means that from 1 gm. of dried pupae around 17,500 units were extracted. By using water instead of alcohol as

TABLE II

Extraction of v^+ Hormone

Weight of pupae	Weight of extract	Units extracted		
		Total	Per gm. extract	Per gm. pupae
gm.	*gm.*			
0.13	0.016	700	43,700	5,380
4.42	0.101	10,500	104,000	2,380
21.5	1.28	266,000	208,000	12,360
50.0	1.32	420,000	318,000	8,400
120.0	5.30	1,484,000	280,000	12,370
160.0	8.05	3,283,000	408,300	20,520
225.0	13.30	2,240,000	168,000	9,970
110.0	5.00	2,100,000	420,000	19,100
110.0	3.86	2,100,000	544,000	19,100
50.0	1.92	1,006,600	524,000	20,130
0.5*	0.096	7,000	72,900	14,000

* Exhaustive extraction with water only.

an extractive, about the same number of units was obtained, but the extracts were less pure.

Some of the best extractions yielded 20,000 units per gram pupae, containing about 2,500 individuals. Consequently in these cases approximately 8 units were obtained from each individual. Fig. 1 shows that 30 units are required to produce the maximum effect, so that one wild type fly must have produced at least 30 units, and perhaps much more. If this figure is accepted, at most 28 per cent of the theoretical amount was recovered from the pupae. This poor recovery might be due to incomplete extraction or to partial inactivation, but in view of the relatively constant yield, it seems probable that the concentration of the hormone in the pupae at any one time does not greatly exceed this extractable amount.

Properties of the v^+ Hormone

With the aims of obtaining some indication of the chemical nature of the hormone and of developing a satisfactory purification procedure, some of its physical and chemical properties were investigated. Unless otherwise stated, the various treatments were carried out on extracts prepared as described above. In most cases the reagents or solvents used were removed by precipitation or distillation and the residual material diluted to a known volume with Ringer's solution. If necessary the treated material or fraction was adjusted to a pH of

TABLE III

Heat Stability of Hormones

Treatment	v^+		cn^+	
	Color	Units	Color	Units*
100°C. for 0.5 hr.	3.0	6.0	3.0	6.0
3.0	3.0	6.0	2.5	4.0
12.0	3.0	6.0	2.5	4.0
36.0	2.5	4.0	1.0	1.0
1 hr. at 100°C.	3.0	6.0	3.5	10.0
120	2.5	4.0	2.5	4.0
140	2.0	2.5	2.0	2.4
160	0.2	0.4	0.1	0.2

* Calculated on assumption that relationship between concentration and eye color is same as with v^+ hormone.

6.0, dried under reduced pressure, and reextracted with alcohol. The alcohol was removed from the extract and the residue dissolved in Ringer's solution. The extracts were then tested for activity as previously described.

Heat Stability.—The stability of the hormones was determined by heating whole pupae in the oven at various temperatures for different periods of time. The treated material was crushed in a few drops of Ringer's solution and fed to test larvae as described by Beadle and Law (1938). This method was sufficiently exact for comparison of the different samples. The results are given in Table III. The

hormones were found to be stable at a temperature of 100°C. for 1 hour. Over much longer periods of time or at higher temperatures the activity of both hormones decreased until at 160°C. practically all the activity was lost in 1 hour. In general the v^+ and cn^+ hormones showed similar behavior towards heat. The destruction of the hormones at higher temperatures was also substantiated by comparing the activity on injection of standard extracts prepared from pupae dried at 100°C. and at 130–140°C. The latter extract contained only one-fourth as much v^+ and cn^+ hormones.

Whereas intact dried pupae, stored at 20–25°C., retained the v^+ hormone in an active state for at least 11 days (tested by feeding), ground-up material slowly decreased in effectiveness until after 11 days it was only one-fourth as active. Such stored ground material, however, seemed toxic for the test larvae, so that the apparent loss of activity may have been due to entirely different effects; *e.g.*, a decreased uptake of the hormone by the larvae. This seems all the more likely because no decrease in activity of hormone extracts, either in solution or in dried form, could be observed over a 4 week period of storage at 20–25°C.

Stability to Acid and Alkali.—Small samples of active v^+ extract were treated with N and 10 N sulfuric acid and sodium hydroxide at 100°C. for 30 minutes. The samples were neutralized, dried under reduced pressure, and extracted with ethyl alcohol. The alcohol was removed and the residue was dissolved in Ringer's solution and tested. A control was treated with N sulfuric acid at room temperature. The results were as follows:

Treatment	Destroyed
	per cent
Control	0
N—H_2SO_4	0
10 N—H_2SO_4	76
N—NAOH	80
10 N—NaOH	100

The v^+ hormone was stable to N acid, largely destroyed by 10 N acid and N alkali, and completely inactivated by 10 N alkali.

Stability to Oxidation.—Beadle and Thimann demonstrated the

necessity of destroying the pupal enzymes before extraction, and suggested that the hormones are inactivated by enzymic oxidation. They also stated that hydrogen peroxide inactivated them. Some of our results led us to suspect that the v^+ hormone might be slowly oxidized and inactivated by the oxygen in the air. We therefore tested the effect of oxygen on active extracts. It was found that bubbling oxygen through such solutions for ½ hour had no inactivating effect. No loss was found at pH values of 4, 6, or 9 or at pH 6 in the presence of a little charcoal as a catalyst, or at 100°C. for ¾ hour. It seems improbable that the substance is oxidized by the oxygen in the air under ordinary conditions.

An attempt was then made to repeat the results of Beadle and Thimann involving treatment with hydrogen peroxide. A sample of extract was treated with hydrogen peroxide in a final concentration of 1 per cent. The solution was boiled for ½ hour. The resulting solution was still active, but since it still contained peroxide, only small amounts could be injected and no quantitative results were obtained. When the excess peroxide was decomposed with freshly precipitated iron oxide the solution was toxic.

These experiments were repeated using a more concentrated extract. After heating the extract with 1 per cent hydrogen peroxide for ½ hour, one sample was freed from peroxide by treatment with a few drops of fresh pupal juice as a source of catalase. After 5 minutes the mixture was heated and centrifuged. The peroxide in the other sample was removed by heating with iron oxide. The sample in which the peroxide had been decomposed with catalase showed no significant loss in activity, but the iron oxide-treated sample was slightly toxic and completely inactive. It was concluded that hydrogen peroxide alone does not oxidize the v^+ hormone, but that iron oxide may act catalytically in making this oxidation possible. It was found that iron oxide also catalyzes oxidation by molecular oxygen. At a temperature of 100°C. in the presence of iron oxide and molecular oxygen, 82 per cent of the hormone activity was lost in ¾ of an hour.

Enzymic Inactivation.—Since the hormone had been shown to be relatively stable towards oxidation, it was thought advisable to investigate its inactivation by the enzymes present in the larvae and pupae. Juice obtained by crushing or grinding fresh pupae or larvae

and filtering, was mixed with concentrated active extract. The solutions were allowed to stand for 15–20 hours on watch glasses at 30°C., then diluted with Ringer's solution to known volumes, sealed in glass tubes, heated, centrifuged, and tested by injection. The results of several experiments are shown in Table IV. In no case using juice from crushed pupae or larvae did inactivation result. However, the juice from wild type or vermilion larvae and wild type pupae obtained by grinding the material thoroughly with finely

TABLE IV

Enzymic Inactivation of v^+ Hormone

Enzyme			Inactivation
Source	Preparation	Treatment	
			per cent
Wild type pupae	Crushed	Heated	0
" " "	"	Unheated	0
" " "	Ground	"	83
Wild type larvae	Crushed	Heated	0
" " "	"	Unheated	0
" " "	Ground	Heated	0
" " "	"	Unheated	95
v bw larvae	"	Heated	0
" " "	"	Unheated	89
Wild type larvae	"	Heated, N_2	8
" " "	"	" , O_2	0
" " "	"	Unheated, N_2	8
" " "	"	" , O_2	62

powdered silica, almost completely inactivated the v^+ hormone. The results indicate that the inactivating enzyme is intracellular and is freed only by grinding the cells thoroughly. Tests for the cn^+ hormone in some of these cases showed that it was similarly inactivated.

Another experiment was made which confirmed the hypothesis of Thimann and Beadle that the inactivation is due to enzymic oxidation. The oxygen was removed from samples of heated and unheated enzyme solutions by bubbling purified nitrogen through them. After ½ hour concentrated active extract was added and the stream of nitrogen continued for ½ hour longer. Two other samples were

similarly treated with oxygen and enzyme solutions. The results of this experiment are also given in Table IV. Only when both oxygen and active enzyme were present did inactivation result.

Solubilities of the v^+ Hormone.—The solubilities of the hormone in various organic solvents were also investigated. The results are in complete agreement with those of other workers (Khouvine and Ephrussi, 1937; Becker, 1937). The substance is soluble in water, in 95 per cent ethyl alcohol, slightly soluble in 95 per cent acetone, and quite soluble in 50 per cent acetone. However, it is insoluble in ether, chloroform, benzene, and absolute acetone under ordinary conditions. Continuous extraction of the dried material, however, showed that it could be slowly extracted with absolute acetone. An

TABLE V

Effect of pH on Extraction with Butyl Alcohol

pH	Hormone extracted	
	Eye color produced	Units
5.0	0.8	560
5.5	1.8	1400
6.0	2.3	2100
6.5	1.8	1400
7.0	0.9	630

active water extract was mixed with anhydrous $CaSO_4$ to a crumbly mass, and the mixture was allowed to set overnight. Continuous extraction of this material with hot absolute acetone for 4 hours removed about 10 per cent of the hormone present.

The solubility of the v^+ hormone in ethyl alcohol suggested the possible use of butyl alcohol in its purification. Samples of active solution were shaken with equal volumes of butyl alcohol at varying pH values. The butyl alcohol layer was evaporated to dryness and dissolved in Ringer's solution. The results are given in Table V. The solubility in butyl alcohol was at a maximum at pH 6.0 but decreased sharply at higher or lower pH values. This indicates that the hormone contains both acidic and basic groups with an isoelectric point at about 6.0.

Continuous extraction with butyl alcohol confirmed this evidence.

A water solution containing v^+ hormone adjusted to successive pH values of 9, 3, and 6 was extracted continuously with butyl alcohol. The extractions were carried out under reduced pressure to avoid possible inactivation at higher temperatures and each extraction was continued for 4 hours. The various extracts and the final extracted residues were freed from butyl alcohol and salts and tested. The original solution contained 105,000 units. The extract at pH 9 contained 5,900 units, that at pH 3 contained 16,100 units, while 44,100 units were extracted at pH 6. The residue contained only 1,750 units. These results showed that the active substance was extracted very slowly at pH 9, slightly more rapidly at pH 3, and quite readily at pH 6.

Precipitation Reactions.—The effect of various precipitants on the hormone extracts was investigated in the hope that some would prove of value in the purification of the active principle. It was found that treatment with lead acetate and ammonium hydroxide precipitated the substance completely. This agrees with the results of Khouvine and Ephrussi. Treatment with barium hydroxide and 4 volumes of ethyl alcohol also precipitated it completely. In this case the activity could be completely restored by decomposing the precipitate with sulfuric acid. Treatment of an alcoholic solution of the hormone with mercuric chloride in alcohol did not precipitate it, and the activity was recovered in the filtrate after removing the excess mercury with H_2S. However, Neuberg's reagent (mercuric acetate and sodium carbonate) in 70 per cent alcohol precipitated the hormone. It could be recovered from the precipitate by treatment with H_2S. This precipitant is assumed to be specific for amino acids and amino acid-like substances.

Molecular Weight of Hormones.—Since the chemical investigations had indicated the amino acid-like nature of the v^+ hormone but had given no information as to the size of the molecule, it was thought profitable to determine the approximate molecular weight. The only method applicable to such a determination of an unisolated substance which is present in small amounts is the diffusion method. A complete discussion of this method including the calculation and evaluation of results and references to the literature is given by Cohen and Bruins (1923). Bruins, Overhoff, and Wolff (1931) later used

this method to estimate the molecular weights of carotin and vitamin A. Went (1928), by using agar blocks for diffusion, modified the method for the determination of the molecular weight of auxin.

Because of its simplicity, the agar block technique was used for the determination of the molecular weight of the eye color hormones. 1½ per cent agar blocks 10 × 10 × 2 mm. were used. Block number 1 (the bottom block in the series) was prepared by adding 1.5 per cent dried agar to a concentrated extract. The other blocks contained 1.5 per cent agar in Ringer's solution. After solidification the blocks were cut to the correct size, and three plain agar blocks piled on the extract-containing block. Care was taken to prevent occurrence of air bubbles between the blocks. Diffusion was allowed to proceed at 25°C. After a definite period the blocks were separated, placed in small vials, cut up, and covered with an equal volume (0.2 cc.) of Ringer's solution. After 15 hours at 30°C. the solutions were removed for testing for the v^+ hormone. It was assumed that complete equilibrium had been attained in that time.

To obtain the most accurate possible determination of the hormone concentration in the test blocks, it was desired to dilute the solutions for testing so that all should be of the same hormone concentration. This procedure avoids any errors which might be introduced through the use of the eye color standards. It was found best to assume the molecular weight and to calculate, from the theoretical distribution for that value, the dilutions of each fraction which should give test solutions of equal hormone concentration. The dilutions were so calculated as to give a color value of 1. Slight differences in color are most easily detectable in this region on the color scale.

The results of several determinations are given in Table VI. The diffusion time and the assumed molecular weight are given for each series. The table gives, for each block, the dilution made, the eye color obtained, the theoretical and found distribution expressed in parts per 10,000, and the experimentally determined value of D_{20}. With an assumed molecular weight of 150 the concentration in the diluted samples decreased markedly from blocks 4 to 1, indicating a higher molecular weight. Similarly, the data show that the molecular weight is higher than 300. However, with the dilutions made assuming a molecular weight of 500, the eye colors produced by the four

fractions were nearly equal and well within the range of experimental error and the limit of accuracy of the method. With the v^+ hormone an average value for all the experiments of $D_{20} = 0.316$ was obtained,

TABLE VI

Molecular Weight of Hormones

Molecular weight	Diffusion time	Block No.	Calculated dilution for equal concentration	Eye color obtained from diluted samples	Distribution of hormone per 10,000 total		Found D_{20}*
					Calculated	Found	
	min.						
v^+ Hormone							
150	60	4	13	2.5	5500	6800	0.28
		3	7	1.0	3200	3000	0.34
		2	2.5	0.2	1100	210	†
		1	0	0.1(?)	220	92	0.42
300	60	4	76	1.3	6100	6700	0.28
		3	37	1.1	3000	2800	0.27
		2	9	0.8	730	490	0.30
		1	0	0.5	80	41	0.34
500	109	4	26	0.8	5516	5300	0.34
		3	15	0.9	3201	3400	†
		2	5	0.9	1063	1100	0.33
		1	0	0.8	214	200	0.30
500	109	4	26	1.0	5516	5600	0.31
		3	15	1.0	3201	3200	0.32
		2	5	0.9	1063	1000	0.32
		1	0	0.6‡	214	130	0.27
cn^+ Hormone							
500	109	4	13	1.0	5516	5500	0.31
		3	7.5	1.0	3201	3200	0.31
		2§	2.5	1.0	1063	1100	0.31

* Average value of D_{20} for v^+ hormone was 0.316; for cn^+ hormone, 0.31.

† Unavoidable experimental errors in determinations made it impossible to calculate significant values of D_{20} in these cases.

‡ Minimum value because of toxicity of fraction.

§ Concentration of cn^+ hormone in block 1 was too low to test.

and the calculated molecular weight of the v^+ hormone was 490. A similar determination for the cn^+ hormone gave the same result, a molecular weight of 500.

Because of the limitations of the method the values obtained are

only approximate. It can be safely assumed, however, that the molecular weights of v^+ and cn^+ hormones are of the same order, with a value between 400 and 600.

DISCUSSION

The investigation has shown that the v^+ hormone is probably a single chemical entity of true hormone-like activity. So far there has been no evidence that more than one substance is involved. That it may rightly be classed as a true hormone is indicated by its high activity. The most active preparation yet obtained has a decided effect on eye color in a concentration of 0.23 γ per fly. 1 gm. of this material would be sufficient to modify the eye color of 4,200,000 flies. There is every reason to believe that the active principle makes up only a small percentage of the dry weight of this material and that it will prove to be very much more active than this figure indicates.

The known properties of the v^+ hormone indicate that it is an amino acid-like compound. Its solubility in water and in ethyl alcohol and its insolubility in other organic solvents resemble the characteristic solubilities of amino acids. The extraction with butyl alcohol furnished evidence to the same effect. The influence of pH on the rate of extraction indicates the presence of both acidic and basic groups with an isoelectric point near pH 6. This behavior is also typical of the neutral amino acids.

Further evidence of its amino acid nature is found in its precipitation with Neuberg's reagent, which is assumed to be specific for amino acids and related compounds. Finally, all active extracts so far obtained give strong positive reactions with ninhydrin (triketohydrindenhydrate), which is one of the most sensitive tests for α amino acids.

Although all the data indicate the amino acid nature of the hormone, it seems probable that it is not a simple amino acid. The substance may be destroyed or inactivated by many treatments which would not affect simple amino acids. The instability of the hormone to heat, and to treatment with acid and alkali illustrate this point. Furthermore, the high molecular weight of 400 to 600 as determined by the diffusion method also eliminates the common simple amino acids from consideration.

The available information does not justify any conclusions as to

the exact chemical nature of the hormone. However, there seem to be three distinct possibilities. It may prove to be a complex single amino acid, a peptide of several amino acids, or it may be a compound of one or more amino acids with other substances. In any case it is probably not a normal constituent of tissue, since its occurrence seems to be rather strictly limited to certain insects. Khouvine and Ephrussi were unable to demonstrate any hormone activity in nitrogenous extracts of sheep brain, while similarly prepared extracts of *Calliphora* were very active. Although yeast is generally supposed to contain most known nitrogenous substances, we have had no indication that it contains this particular amino acid-like hormone.

Mention should be made here of the fact that all the data available indicate the very close chemical relationship of the v^+ and cn^+ hormones. No differences in their chemical properties have been found during this investigation. This substantiates the views of Ephrussi and Beadle regarding the interrelationship of the two hormones.

SUMMARY

The substance inducing the production of pigment in the eyes of vermilion brown mutants of *Drosophila melanogaster* has been shown to be a relatively stable chemical entity possessing true hormone-like activity.

A simple method for obtaining hormone solutions has been developed involving extraction of dried wild type *Drosophila* pupae with ethyl alcohol and water.

A logarithmic proportionality has been found to exist between the amount of hormone and the induced eye color. This relationship provides a simple method for the quantitative determination of hormone concentration in given extracts.

Larvae and pupae of *D. melanogaster* contain an intracellular enzyme which inactivates the hormone in the presence of molecular oxygen. The hormone is not oxidized under ordinary conditions with either molecular oxygen or hydrogen peroxide.

The hormone has been found to be an amphoteric compound with both acidic and basic groups and with a molecular weight between 400 and 600.

The solubility and precipitation reactions of the hormone suggest

its amino acid-like nature. However, the instability to heat, acid, and alkali, and its rather restricted occurrence indicate a rather complex specific structure.

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